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Receptor-Controlled Phosphorylation of α_1 Soluble Guanylyl Cyclase Enhances Nitric Oxide-Dependent Cyclic Guanosine 5′-Monophosphate Production in Pituitary Cells

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It is generally accepted that G protein-coupled receptors stimulate soluble guanylyl cyclase (sGC)-mediated cGMP production indirectly, by increasing nitric oxide (NO) synthase activity in a calcium- and kinase-dependent manner. Here we show that normal and GH_3 immortalized pituitary cells expressed $\alpha_1\beta_1$-sGC heterodimer. Activation of adenylyl cyclase by GHRH, pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal peptide, and forskolin increased NO and cGMP levels, and basal and stimulated cGMP production was abolished by inhibition of NO synthase activity. However, activators of adenylyl cyclase were found to enhance this NO-dependent cGMP production even when NO was held constant at basal levels. Receptor-activated cGMP production was mimicked by expression of a constitutive active protein kinase A and was accompanied with phosphorylation of native and recombinant α_1-sGC subunit. Addition of a protein kinase A inhibitor, overexpression of a dominant negative mutant of regulatory protein kinase A subunit, and substitution of Ser_{107}-Ser_{108} N-terminal residues of α_1-subunit with alanine abolished adenylyl cyclase-dependent cGMP production without affecting basal and NO donor-stimulated cGMP production. These results indicate that phosphorylation of α_1-subunit by protein kinase A enlarges the NO-dependent sGC activity, most likely by stabilizing the NO/$\alpha_1\beta_1$ complex. This is the major pathway by which adenylyl cyclase-coupled receptors stimulate cGMP production. (*Molecular Endocrinology* 18: 458–470, 2004)

THE L-ARGININE-NITRIC OXIDE (NO) signaling pathway is involved in the regulation of various cellular events, including activation of soluble guanylyl cyclase (sGC), an enzyme that catalyzes the formation of cGMP (1). In physiological situations, NO is derived by NO synthases (NOS). Two of these enzymes, neuronal NOS (nNOS) and endothelial NOS (eNOS), operate in a calcium-dependent manner, whereas inducible NOS (iNOS) is fully active at basal intracellular calcium concentrations (2). The structures of four sGC subunits, termed α_1, α_2, β_1 and β_2, have been identified to date (3–6). The $\alpha_1\beta_1$ and $\alpha_2\ \beta_1$ heterodimers exhibit high and comparable catalytic activities, the $\alpha_1\beta_2$ heterodimer is less active, and none of the homodimers is functional (7, 8). The C termini of these subunits are sufficient for generating cGMP, whereas the N termini account for NO responsiveness (9, 10). The binding of NO to the heme iron leads to conformational changes in the enzyme, resulting in a severalfold increase in cGMP production (11, 12). The importance of both sGC subunits for heme coordination and NO sensitivity has been confirmed in experiments with mutant heterodimers, in which the nonconserved N-terminal 131-α_1 and 64-β_1 residues were deleted (13). An NO-independent regulatory site on sGC has also been identified (14).

G protein-coupled receptors that facilitate calcium mobilization from intracellular stores and/or calcium influx through plasma membrane channels are believed to stimulate sGC through calcium-dependent NOS (15). In accordance with this view, receptors in pituitary cells that stimulate adenylyl cyclase also increase intracellular calcium and cGMP production, whereas receptors that inhibit adenylyl cyclase also inhibit spontaneous calcium transients and decrease cGMP production (16–18). However, several lines of evidence suggest that these receptors in pituitary cells also modulate sGC activity independently of the status of intracellular calcium. Parallelism in the receptor-mediated up- and down-regulation of cAMP and cGMP production further suggests that cAMP could translate the actions of these receptors on cGMP production (18). The effects of adenylyl cyclase-coupled

Abbreviations: CMI, 4-Cyano-3-methylisoquinoline; DPTA, 3,3′-(hydroxynitrosohydrazino]bis-1-propanamine; eNOS, endothelial NOS; IFN, interferon; iNOS, inducible NOS; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NO, nitric oxide, NOS, NO synthase; nNOS, neuronal NOS; PACAP, pituitary adenylate cyclase activating polypeptide; PKA, cAMP-dependent protein kinase A; sGC, soluble guanylyl cyclase; VIP, vasoactive intestinal peptide.

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receptors and forskolin, an activator of adenylyl cyclase, on cGMP production are not unique for pituitary cells, but are also observed in other cell types, including pineal gland (19).

Changes in the endogenous phosphodiesterase activity by increase in cAMP production and/or activation of cAMP-dependent protein kinase A (PKA) could account for receptor-induced cGMP accumulation (20, 21). Our earlier study, however, showed that inhibition of phosphodiesterase activity with a cocktail of blockers does not inhibit, but amplifies, agonist-stimulated cGMP accumulation (18). These observations do not exclude a role of cAMP/PKA in control of phosphodiesterase activity in pituitary cells, but indicate that G_s-coupled receptors stimulate *de novo* cGMP production independently of the status of these enzymes. In general, the stimulatory action of adenylyl cyclase-coupled receptors on cGMP production could be mediated indirectly, by phosphorylating eNOS, which makes this enzyme operative at basal intracellular calcium (22–25), or directly, by phosphorylating sGC (26).

To address this issue, we used the primary culture of pituitary cells and GH_3 immortalized cells. Pituitary cells express several G_s-coupled receptors, including GHRH receptor (27), pituitary adenylate cyclase activating polypeptide (PACAP)-specific PVR1 receptor, and PACAP-vasoactive intestinal peptide (VIP)-sensitive PVR3 receptor (28). GH_3 immortalized pituitary cells express PVR3 receptors (29), whereas the expression of GHRH receptor in these cells has been questioned (30–32). In pituitary and GH_3 cells, we identified NOS subtypes and $\alpha\beta$-sGC subunits expressed. In addition to GHRH, we stimulated cells by PACAP 27, VIP, and forskolin, an adenylyl cyclase activator, and examined the role of PKA by expressing constitutive active and dominant-negative forms of enzyme in GH_3 cells. Our results indicate that PKA-mediated phosphorylation of sGC α-subunit, rather than increase in NOS activity, accounts for receptor-controlled cGMP production and that substitution of Ser_{107}-Ser_{108} N-terminal residues of α_1-subunit with alanine abolished this stimulatory action.

RESULTS

G_s-Coupled Receptors and cGMP Production

The presence of GHRH receptor-specific mRNA in pituitary and GH_3 cells was analyzed by RT-PCR using the PCR primer pairs earlier defined by Mayo (33) and Zeitler *et al*. (34). As shown in Fig. 1A, primers 2 and 3 cover the 5′-portion of the receptor sequence, and primers 4 and 5 spans the 3′-portion of receptor including the carboxyl terminus. In both cell types, amplification of RNA with three pairs of primers resulted in identical products of expected sizes (single products for pairs 2, 3, and 5 and double product for pair 4). Furthermore, in GH_3 cells with attenuated phosphodiesterase activity, GHRH induced a concentration-dependent increase in cAMP accumulation (Fig. 1B). These results indicate that, in our experimental conditions, GH_3 cells also express functional GHRH receptors.

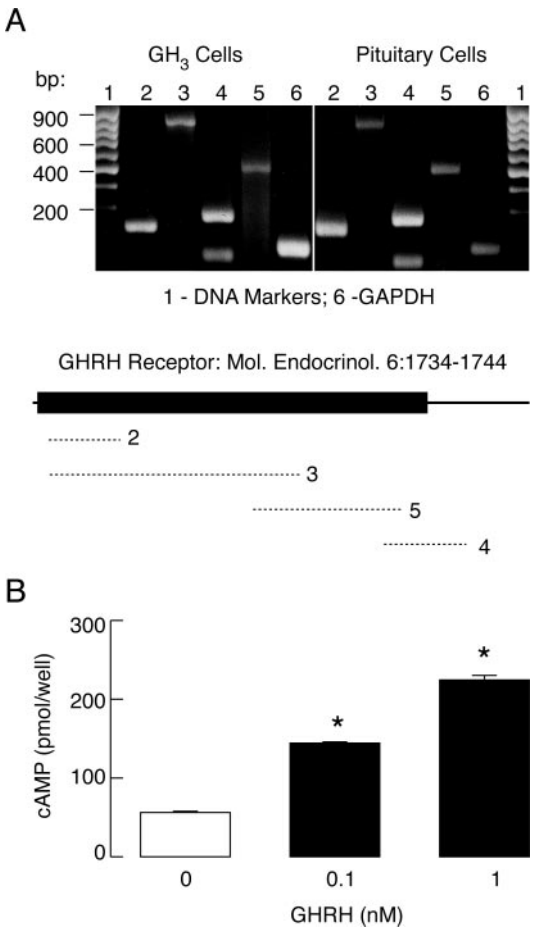

Fig. 1. Expression of Transcripts for GHRH Receptors in Pituitary and GH_3 Cells

A, RT-PCR analysis of GHRH receptor mRNA expression in GH_3 cells (*left upper panel*) and pituitary cells (*right upper panel*) using primer pairs 2–5. Schematic representation of PCR primer pairs used for amplification of GHRH receptor transcripts in pituitary and GH_3 cells is shown in *bottom panel. Black box* indicates GHRH coding sequence, *flanking lines* indicate 5′- and 3′-untranslated regions, and *dotted lines* indicate regions amplified by primer pairs. B, GHRH stimulates cAMP production in GH_3 cells in physiological concentrations. If not otherwise specified, in these and following figures, endogenous phosphodiesterase activity was attenuated by 1 mM 3-isobutyl-1-methylxanthine and cells were incubated for 60 min. Data shown are means ± SEM from sextuplicate determinations in one of three similar experiments. *Asterisks* indicate significant differences between controls and treated cells, $P < 0.05$.

In mixed populations of pituitary cells with attenuated phosphodiesterase activity, GHRH induced a concentration-dependent increase in cGMP accumulation (Fig. 2A, *left panel*). GH_3 cells also responded to GHRH stimulation with a progressive increase in cGMP production (Fig. 2A, *right panel*). Forskolin, an

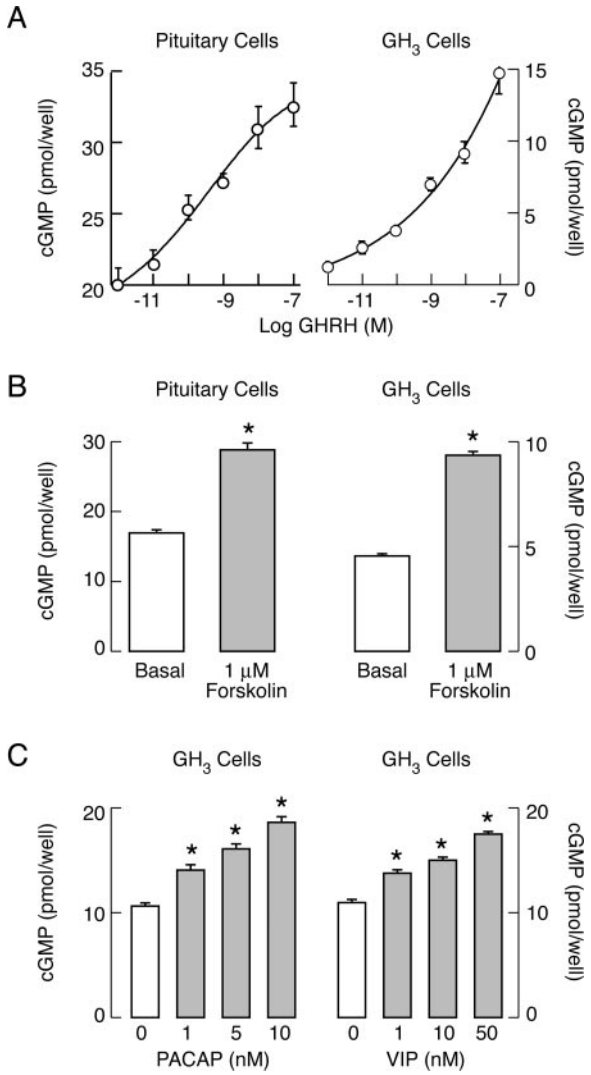

Fig. 2. Receptor- and Forskolin-Stimulated cGMP Production in Pituitary and GH_3 Cells

A, Concentration dependence of GHRH on cGMP production in pituitary cells (*left panel*) and GH_3 cells (*right panel*). B, Forskolin-induced cGMP accumulation. C, Concentration dependence of PACAP (*left panel*) and VIP (*right panel*) on cGMP accumulation. In panels B and C, *asterisks* indicate significant differences between controls (basal, 0) and treated cells, $P < 0.05$.

activator of adenylyl cyclase, mimicked the action of GHRH on cGMP accumulation in both cell types (Fig. 2B). The addition of two other agonists in GH_3 cells, VIP and PACAP, also resulted in a concentration-dependent accumulation of cAMP (Table 1) and cGMP (Fig. 2C). These results indicate that receptor- and non-receptor-mediated activation of adenylyl cyclase leads to increase in cGMP production.

Dependence of cGMP Production on PKA Activity

Agonist-stimulated sGC activity in pituitary cells was abolished by 4-cyano-3-methylisoquinoline (CMI), a

Table 1. Effects of PACAP 27 and VIP on cAMP Production (pmol/well) in GH_3 Cells

Agonist Concentration (nM)	PACAP 27	VIP
0	8.8 ± 0.4[a]	8.7 ± 0.2
1	222 ± 10	175 ± 5
10	289 ± 8	211 ± 10
50	448 ± 22	310 ± 9

[a] Data shown are means ± SEM from sextuplicate determinations.

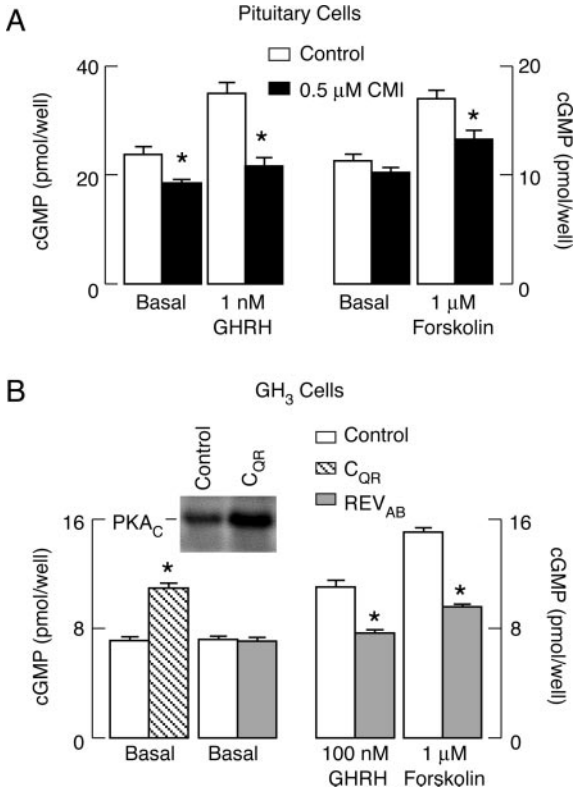

Fig. 3. Dependence of GHRH- and Forskolin-Induced cGMP Production on PKA Pathway

A, Attenuation of GHRH- and forskolin-induced cGMP production in pituitary cells by CMI, a PKA inhibitor. B, Stimulation of cGMP production by constitutively active PKA (C_{QR}) and inhibition of GHRH- and forskolin-induced cGMP production by dominant negative mutant of regulatory PKA subunit (REV_{AB}) in GH_3 cells. *Inset* indicates the expression of catalytic PKA (PKA_C) in controls and cells transfected with C_{QR}. *Asterisks* indicate significant differences between the pairs, $P < 0.05$.

specific inhibitor of PKA (Fig. 3A, *left panel*). Forskolin-induced increase in cGMP levels in these cells was also inhibited by CMI (Fig. 3A, *right panel*), suggesting that PKA mediates the action of cAMP on cGMP production. More definite support to this hypothesis was obtained in experiments with constitutive active and dominant negative PKA constructs (35, 36). In GH_3 cells transfected with a construct of constitutively ac-

tive PKA, an increase in expression of PKA catalytic subunit was observed (Fig. 3B, *left panel inset*) and was accompanied with a significant increase in cGMP levels (Fig. 3B, *main left panel*). Furthermore, GHRH- and forskolin-induced cGMP production was inhibited in GH_3 cells expressing the dominant negative regulatory subunit of the enzyme (Fig. 3B, *right panel*), whereas basal enzyme activity was not affected (Fig. 3B, *left panel*).

Dependence of cGMP Production on NO Levels

The potential relevance of PKA-dependent phosphorylation of NOS on agonist-stimulated cGMP production was tested in both cell types. In GH_3 cells, Western blot analysis revealed the presence of nNOS, no expression of eNOS, and the lack of iNOS expression in response to stimulation with lipopolysaccharide + interferon-γ (LPS+IFN) (Fig. 4A). In these cells, activation of adenylyl cyclase by GHRH (data not shown) and forskolin (Fig. 4B) induced a small increase in phosphorylation of nNOS, but it was not accompanied by changes in NO levels (Fig. 4C). Forskolin was also ineffective in elevating NO levels in GH_3 cells (Fig. 4C).

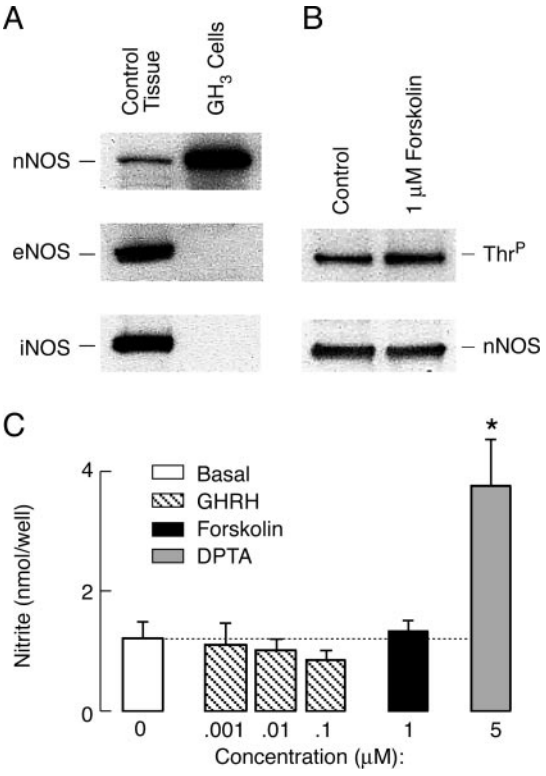

Fig. 4. Expression, Phosphorylation, and Activity of nNOS in GH_3 Cells

A, Western blot analysis of NOS expressed in GH_3 cells. Control tissue: nNOS, brain; eNOS, aorta; iNOS, pituitary cells treated with LPS+IFN overnight. B, Forskolin-induced phosphorylation of nNOS, estimated by an antiphosphothreonine monoclonal antibody (*upper panel*). Blot was stripped and tested for protein content by a polyclonal anti-nNOS antibody (*bottom panel*). C, The lack of effects of GHRH and forskolin on NO production in GH_3 cells. *Asterisk* indicates significant differences between basal and DPTA-stimulated cells.

On the other hand, mixed populations of anterior pituitary cells constitutively express nNOS and eNOS (17), as well as iNOS in response to LPS+IFN stimulation (Fig. 4A). The expression of iNOS did not affect basal cAMP production, but significantly decreased GHRH-induced cAMP production (Fig. 5A). In contrast, basal cGMP production was elevated in iNOS-expressing cells, and GHRH further stimulated sGC activity in a manner comparable to that observed in control cells, but with higher amplitude of response (Fig. 5B). During a 2-h incubation, 100 nM GHRH and 1 μM forskolin increased NO levels in controls (Fig. 5C, *left panel*) and iNOS-expressing cells (*right panel*). Increase in NO production was lost in cells treated with 1 nM and lower concentrations of GHRH (data not shown). The time course study in iNOS-expressing cells also revealed the lack of significant differences in NO levels between controls and GHRH/forskolin-treated cells after 30 and 60 min of incubation. As shown in Fig. 5D, there was a linear relationship between NO and cGMP levels in untreated and GHRH/forskolin-treated cells, but cGMP levels were enlarged in treated cells at all three time points. These experiments confirmed that the rise in NO production above basal production is not essential for GHRH action.

To test the relevance of basal NO production on GHRH action, pituitary cells were treated with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS. This treatment did not affect basal and GHRH-induced cAMP production (Fig. 6A), but inhibited basal and GHRH-stimulated cGMP production in a concentration-dependent manner (Fig. 6B). At high inhibitor concentrations, GHRH-stimulated cGMP production was completely abolished. As shown in Fig. 6C, L-NAME decreased endogenous NO production in both groups, but no difference in NO levels was observed in controls and GHRH-stimulated cells. The stimulatory actions of GHRH and forskolin were also abolished in GH_3 cells treated with L-NAME (data not shown).

We further examined the action of GHRH in pituitary cells with elevated NO levels. In these experiments, the endogenous NOS activity was blocked by 1 mM L-NAME, and cells were stimulated with 3,3′-(hydroxynitrosohydrazino]bis-1-propanamine (DPTA), a slow releasable NO donor. Figure 6D shows that DPTA increased NO and cGMP levels in pituitary cells in a concentration-dependent manner. In these experimental conditions, GHRH further stimulated cGMP production (Fig. 6E). As expected, no difference in NO levels were observed in controls and GHRH-stimulated cells in the presence of DPTA (Fig. 6F). These data confirm the hypothesis that adenylyl cyclase-coupled GHRH receptors in pituitary cells can stimulate cGMP production through the pathways other than increasing the NOS activity, but the basal NO

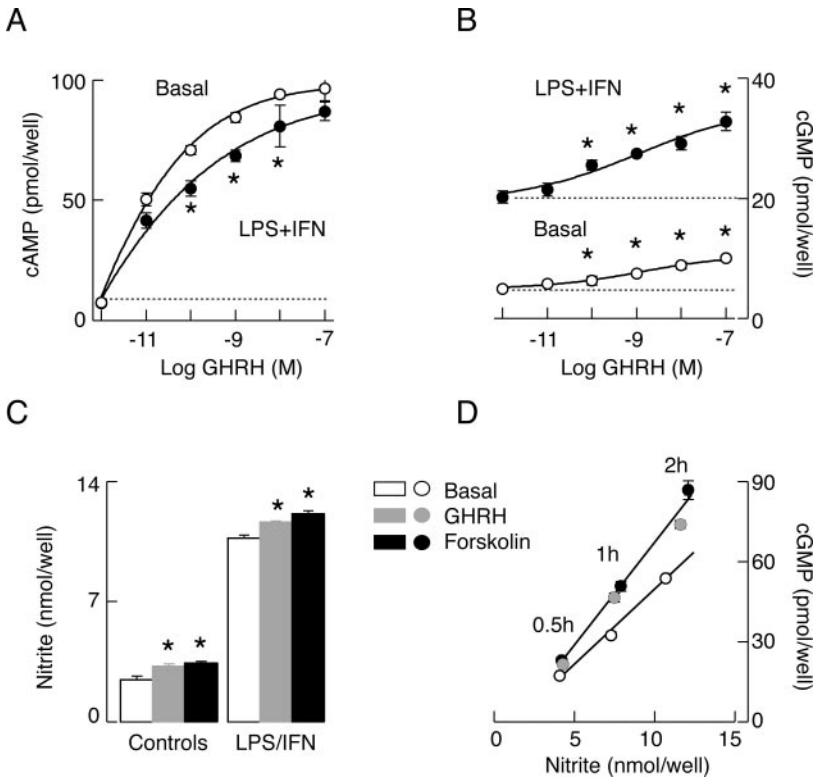

Fig. 5. GHRH and Forskolin-Induced Signaling in Pituitary Cells with and without Expressed iNOS

A and B, Concentration-dependent effects of GHRH on cAMP (A) and cGMP (B) production. C, Comparison of NO levels in controls (*left panel*) and iNOS-expressing cells (*right panel*) after 2 h incubation. To express iNOS, cells were treated with LPS+IFN. D, Correlation between NO and cGMP levels in controls and GHRH/forskolin-treated cells during 0.5-, 1-, and 2-h incubation. *Asterisks* indicate significant differences among pairs (A), controls *vs.* GHRH-stimulated cells (B), and controls *vs.* GHRH (100 nM) and forskolin (1 μM)-stimulated cells (C).

production is needed for the development of stimulatory PKA action on sGC activity.

Expression and Phosphorylation of sGC Subunit

RT-PCR analysis indicated the expression of mRNA for α_1- and β_1-subunits of sGC in normal and immortalized pituitary cells (Fig. 7A). The expression of α_1- and β_1-proteins was confirmed by Western blot analysis using a polyclonal antibody common for both subunits. As shown in Fig. 7B, *left panel*, the β_1-lane was more robust, suggesting that the antibody used was more sensitive for this particular subunit, or that α_1-subunit was less expressed and thus represented a limiting factor in formation of functional sGC heteromers. Consistent with the second hypothesis, overexpression of recombinant rat α_1-subunit in GH_3 cells led to severalfold increase in basal cGMP production. In such cells, forskolin (Fig. 7C) and GHRH (Fig. 7D) further increased cGMP production. We also constructed α_1-subunit with V_5 epitope attached to the C terminus, and the expression of this subunit was confirmed by Western blot analysis using a specific monoclonal antibody against this sequence (Fig. 7B, *right panel*). When expressed in GH_3 cells under identical conditions, no differences in basal and forskolin-stimulated cells were observed between cultures expressing α_1 and α_1-V_5 sGC subunits (Fig. 7C, *left vs. right panel*), indicating that the attachment of residues at the C terminus did not affect the activity of enzyme. In GH_3 cells overexpressing α_1-subunit, GHRH-induced stimulation of sGC was abolished by CMI and by coexpression of the dominant negative PKA mutant (Fig. 7D, *left panel*). On the other hand, no change in cAMP production was observed (Fig. 7D, *right panel*).

To show phosphorylation by PKA, pituitary cells and GH_3 cells were treated with medium 199 (controls), GHRH, and GHRH + CMI, and immunoprecipitation was done using a polyclonal anti-sGC antibody (Fig. 8, A and B). An antiphosphoserine antibody revealed the phosphorylation of α_1-subunit by GHRH in normal (Fig. 8A, *left panel*) and immortalized (Fig. 8B, *left pane*) pituitary cells, which was abolished in cells treated with CMI. As shown in Fig. 8, A and B, lanes 2, the levels of sGC content in controls (I), GHRH-treated (II), and GHRH + CMI-treated cells (III), estimated by a polyclonal anti-sGC antibody, was comparable. The mean ± SEM values for phosphorylation of α_1-subunit derived from three experiments are shown in Fig. 8, A and B, *right panels*. No detectable effects of GHRH and forskolin were observed on β_1-subunit (data not

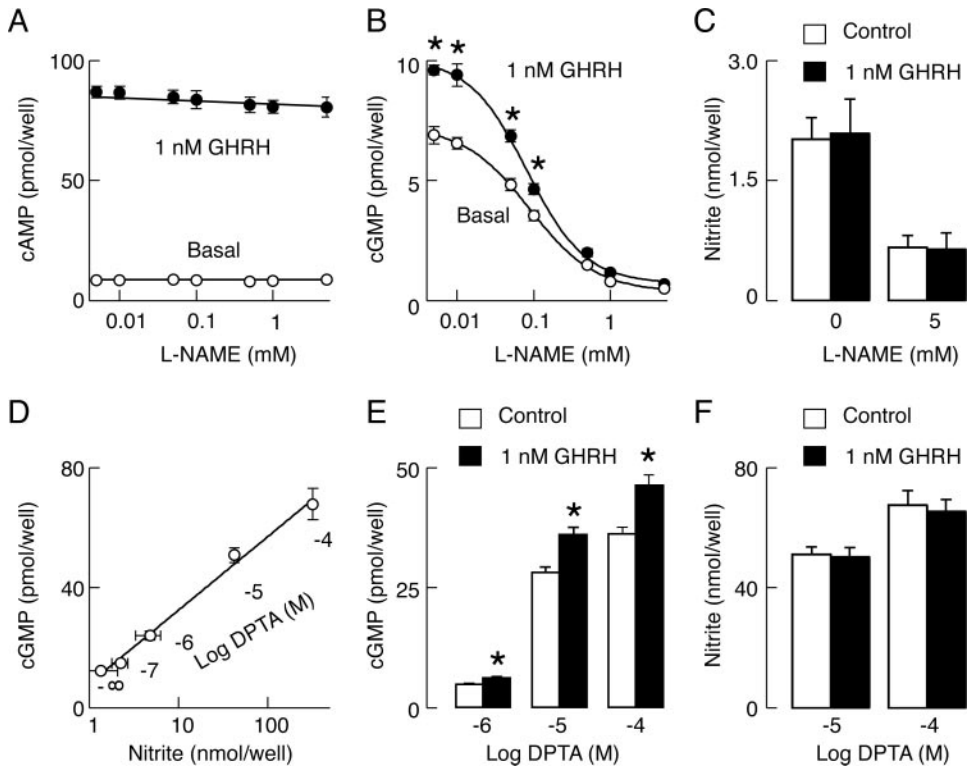

Fig. 6. Dependence of GHRH-Induced cGMP Production on Basal NO Levels in Pituitary Cells

A, The lack of effects of L-NAME, a specific inhibitor of NOS, on basal and GHRH-stimulated cAMP production. B, Concentration-dependent effects of L-NAME on basal and GHRH-induced cGMP production. C, Effects of L-NAME on NO production in controls and GHRH-stimulated cells. D, Correlation between NO levels and cGMP production in cells stimulated with increasing doses of DPTA, an NO donor, in the presence of 1 mM L-NAME. E, Concentration dependence of DPTA on basal and GHRH-stimulated cGMP production in cells with inhibited endogenous NOS activity by L-NAME. F, The lack of effects of GHRH on NO production in cells stimulated with DPTA. In all panels, *asterisks* indicate significant differences between the pairs, $P < 0.05$.

shown). Not only native but the recombinant α_1-V_5 sGC expressed in GH_3 cells was also phosphorylated. As shown in Fig. 8C, *left panel*, line 1, forskolin increased phosphorylation of α_1-V_5 subunit, which was immunoprecipitated by the anti-V_5 antibody and tested for phosphorylation with the antiphosphoserine antibody. The level of expression of α_1-V5 sGC was comparable in controls (I) and 1 μM forskolin (IV).

To search for the PKA phosphorylation site at α_1-subunit, we selected six residues that match the RXS* structure, which is the most common phosphorylation motif for PKA. The serine residues were substituted with alanine, and mutants were expressed in GH_3 cells. As shown in Fig. 9A, the three N-terminal residues, S^{107}, S^{108}, and S^{241}, are in a close proximity of earlier characterized β_1-H^{105}, which operates as a heme ligand, and β_1-C^{78} and β_1-C^{241}, which are important in heme binding (39, 40). α_1-S^{359} and α_1-S^{360} are probably in a region responsible for subunit dimerization, whereas α_1-S^{541} is located in C-terminal catalytic domain. All single-residue mutations, as well as the double mutant, did not affect basal and DPTA-stimulated cGMP production, suggesting that NO binding and activation of enzyme are preserved (Fig. 9B). Furthermore, in four mutations, S241A, S359A, S360A, and S541A, the GHRH-induced increase in cGMP production was comparable to that observed in controls. However, in two single mutants, S107A and S108A (shown by *gray bars* in Fig. 9C, *upper panel*), and a double mutant S107A+S108A (shown as a *black bar*), GHRH was unable to increase cGMP production above the basal level (Fig. 9C, *upper panel*). The lack of effects of GHRH in these mutants was not due to decreased cAMP production (*bottom panel*). In addition, the expression level of these three mutants was comparable to that observed in cells transfected with α_1-V_5 sGC (Fig. 10A). These data indicate that α_1-subunit is critical for the action of AC-coupled receptors on sGC activity.

The lack of GHRH action on sGC activity in S107A and S108A mutants indicate that it might represent the phosphorylation site for PKA, or that intramolecular changes triggered by phosphorylation site on α_1-subunit are affected by these mutations. To dissociate between these possibilities, we generated S107D, S108D, and S107D+S108D α_1-V_5 sGC mutants to determine whether these mutants act as phospho mimics. As shown in Fig. 10A, *bottom panel*, α_1-V_5

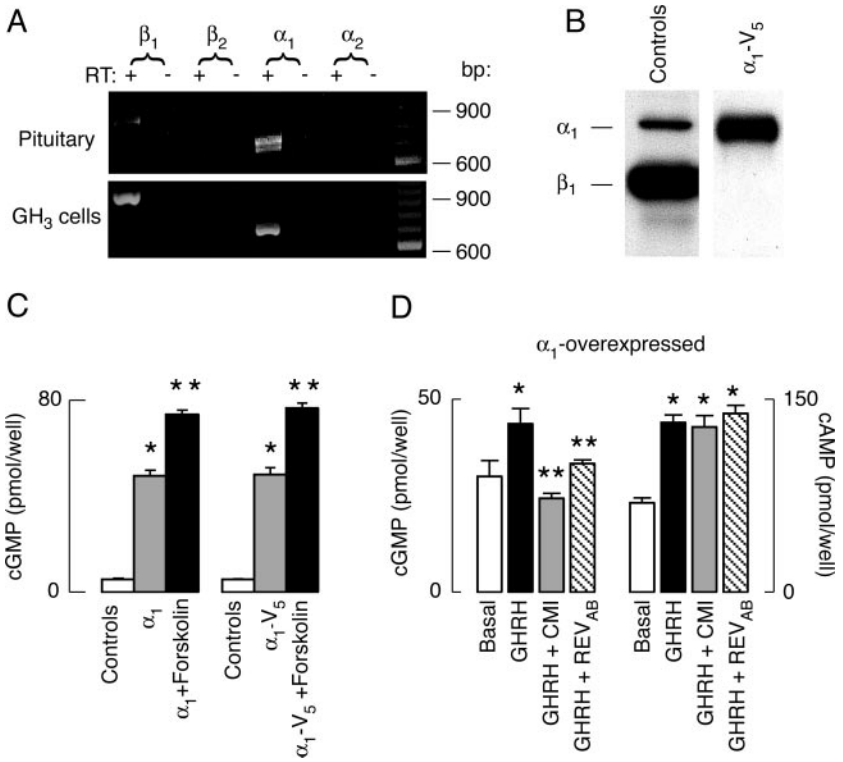

Fig. 7. Characterization of sGC Subunit Expression and Function in Pituitary Cells

A, RT-PCR analysis of expression of native α- and β-subunit transcripts in pituitary and GH_3 cells. RT−, negative controls with PCR conducted using first-strand cDNA samples without RT. B, Western blot analysis of native α_1- and β_1-subunit expression in pituitary cells (*left panel*) and α_1-V_5 expression in GH_3 cells (*right panel*). The native α_1- and β_1-subunits were identified using a common antibody and α_1-V_5 was identified using specific anti-V_5 epitope antibody. C, Effects of transient expression of α_1-sGC (*left panel*) and α_1-V_5-sGC (*right panel*) in GH_3 cells on basal and forskolin (1 μM)-induced cGMP production. *Asterisks* indicate significant differences between controls and α_1/α_1-V_5 (*) and between α_1 and α_1-V_5, and α_1 and α_1-V_5+forskolin groups (**). D, Inhibition of GHRH (100 nM)-induced cGMP production by CMI (0.5 μM) and dominant-negative PKA (REV_{AB}) in α_1-overexpressing GH_3 cells. *Asterisks* indicate significant differences compared with basal cGMP production (*) and GHRH *vs.* GHRH+CMI/GHRH+REV_{AB} groups (**).

sGC and three mutants were expressed at comparable levels in GH_3 cells, which resulted in a severalfold increase in basal cGMP production. However, there was no difference in activities of alanine and aspartic acid mutants (Fig. 10B).

To examine the relationship between NO levels and phosphorylation of α_1-V_5-sGC subunit and S107A+S108A and S107D+S108D mutant enzymes, the endogenous NO production in GH_3 cells was silenced by 1 mM L-NAME, and cells were stimulated with increasing DPTA concentrations in the absence (Fig. 10C) and presence of 1 μM forskolin (Fig. 10D). In the absence of forskolin, DPTA induced a dose-dependent increase in cGMP production in a comparable manner in controls and all mutants, whereas forskolin enlarged it in α_1-V_5 and S107D+S108D mutant, but not in S107A+S108A mutant. The effectiveness of forskolin to amplify NO-dependent cGMP production increased progressively with an increase in NO levels. Because the endogenous NO production was abolished and DPTA-derived NO levels were highly comparable in forskolin-treated and untreated cells (not shown), these results suggest that phosphorylation of α_1-subunit of sGC enhances the effectiveness of NO in activating sGC and that substitution of serine107 and/or 108 with alanine abolishes this stimulatory action.

DISCUSSION

Heteromeric $\alpha\beta$ sGC dimer is a recognized receptor for NO and formation of cGMP in response to a wide variety of agents, including hormones and neurotransmitters acting through G protein-coupled receptors. Stimulation of sGC is especially well established for receptors that signal through phospholipase C- and adenylyl cyclase-dependent pathways (15). Initially, it was believed that calcium mediates the coupling of these receptors to sGC by stimulating eNOS and nNOS (15). Both enzymes are also expressed in normal pituitary cells (17), whereas GH_3 cells express only nNOS (37–40), and their activation by spontaneous voltage-gated calcium influx is critical for basal cGMP

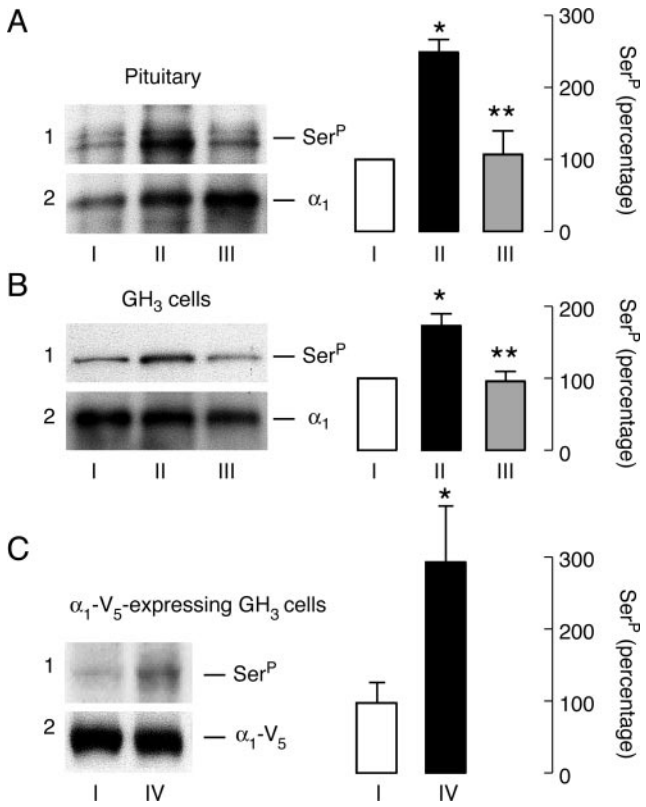

Fig. 8. Phosphorylation of α_1-Subunit in Pituitary and GH_3 cells

A–C (*left panels*), GHRH-induced phosphorylation of native α_1-subunit in pituitary (A) and GH_3 cells (B), and GH_3 cells overexpressing α_1-V_5-sGC (C). Immunopreciptiation was done using a polyclonal anti-sGC antibody (A and B), and specific anti-V_5 epitope antibody (C), and phosphorylation was estimated by an antiphosphoserine monoclonal antibody (*left panels*, lanes 1). Blots were stripped and tested for protein content by polyclonal anti-sGC and anti-V_5 epitope antibodies (lanes 2). A–C (*right panels*), Phosphorylation was expressed as percentage of the phosphorylated control (n = 3). I, Basal; II, 100 nM GHRH-stimulated cells; III, 100 nM GHRH + 0.5 μM CMI-treated cells; IV, 1 μM forskolin-treated cells. *, Significant differences between basal and GHRH/forskolin-treated cells; and **, between GHRH and GHRH+CMI-treated cells. CMI was added 15 min before GHRH.

production (17). However, more recent findings have indicated that activation of G_s protein-coupled GHRH, corticotropin-releasing factor, and TRH receptors also leads to an increase in cGMP levels in pituitary cells, in which calcium signaling was abolished (18).

The ability of G_s-coupled receptors to increase cGMP levels in a calcium-independent and cAMP-dependent manner is compatible with findings that phosphorylation of phosphodiesterases by protein kinases A and G stimulates or inhibits the enzyme activity, depending on the subtype of enzyme (20, 21). However, the stimulatory action of G_s-coupled receptors on cGMP is preserved in pituitary and GH_3 cells bathed in medium containing a cocktail of inhibitors (18), indicated that other pathway accounts for increase in cGMP levels.

The other obvious targets for phosphorylation are NOS enzymes. The serine/threonine protein kinase Akt/protein kinase B-induced phosphorylation of eNOS is well established (23–25). One report also suggests that eNOS from endothelial cells is activated upon phosphorylation by cyclic nucleotide-dependent protein kinases (22). The presence of eNOS in mixed pituitary cells (17) and GHRH and forskolin-induced stimulation of NO production during the sustained incubation shown here support the hypothesis that phosphorylation of this enzyme enhances the enzyme activity. An earlier study, based on GH secretion, also suggested that GHRH stimulates the synthesis of NO at least partially through cAMP (41). However, two lines of evidence indicate that it is not the major pathway by which GHRH stimulates cGMP production. First, GHRH also stimulated cGMP production in pituitary cells with inhibited NOS activity when the NO levels were clamped by slowly releasable NO donor. Second, GH_3 cells exclusively express nNOS, and this enzyme is also phosphorylated in forskolin-treated cells, but we were unable to see any increase in NO levels, and others observed a significant inhibition of enzyme activity in phosphorylated state (42, 43).

Our study revealed a novel mechanism by which G protein-coupled receptors stimulate sGC activity at basal NO levels. This mechanism, summarized in Fig. 11, requires the coupling of receptors to adenylyl cy-

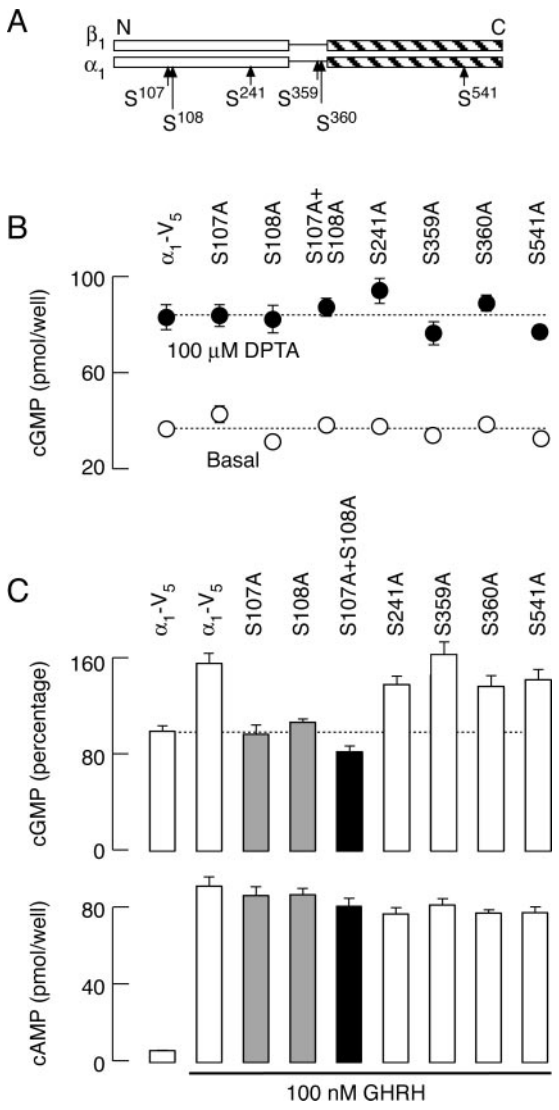

Fig. 9. Effects of Mutations at α_1-sGC Subunit on Enzyme Activity

A, Schematic representation of point mutations in α_1-subunit. B, NO responsiveness of mutant sGC expressed in GH_3 cells. C, Characterization of GHRH-induced cGMP (*upper panel*) and cAMP (*bottom panel*) production in GH_3 cells expressing α_1-sGC mutants. Notice that GHRH-induced sGC activity was completely abolished only in S107A and S108A (*upper panel, gray bars*), and S107A+S108A mutants (*black bar*), and that cAMP production measured in the same cells was not affected (*bottom panel*).

clase, stimulation of cAMP production, and activation of PKA, which phosphorylates sGC. This, in turn, facilitates the $\alpha_1\beta_1$-sGC activity. Down-regulation of basal nNOS activity by L-NAME blocks the stimulatory action of PKA, and elevation of intracellular NO increases it. The overexpression and site-directed mutagenesis indicated that the α_1-subunit is phosphorylated by PKA, and additional studies are needed to identify the phosphorylated residue. The results further indicate that protein kinase A-mediated phosphorylation of α_1-subunit enhances the NO-mediated enzyme activation. This observation is consistent with recent data showing that sGC activity is enlarged by the addition of two compounds, YC-1 (44) and Bay 41–2272 (14), without an increase in NO production, presumably through inhibition of NO-dependent deactivation of sGC (45). In that respect, the PKA-mediated phosphorylation of α_1-sGC represents a physiological mechanism by which the enzyme is activated at constant NO production.

Earlier published data suggested that GHRH receptors are not expressed in GH_3 cells (30–32). In our experimental conditions, however, GH_3 cells expressed transcripts for GHRH receptors and responded to picomolar concentrations of GHRH with a significant increase in cAMP production, suggesting the presence of functional receptors. Although additional studies are needed to clarify this hypothesis, from the point of these investigations it is not a critical issue, because the action of GHRH receptor on sGC is not unique for this receptor but represents a common mechanism by which other G_s-coupled receptors enhance sGC activity. As discussed above, TRH and corticotropin-releasing factor also stimulate cGMP production in adenylyl cyclase-dependent manner (18). Furthermore, here we show that two other agonists from the PACAP/glucagon superfamily, PACAP 27 and VIP, also stimulated cGMP production in GH_3 cells. The action of these two agonists was probably mediated by PVR3 receptors, which are expressed in GH_3 cells (29). Finally, forskolin mimicked the action of GHRH, VIP, and PACAP 27 on phosphorylation of native and recombinant α_1-sGC activity.

Both cAMP and cGMP have important and specific roles in control of electrical activity, calcium signaling, and secretion in pituitary cells, and thus their cross-talk could be important for synchronization of cellular functions. In that respect, stimulation of sGC activity by adenylyl cyclase receptors provides an effective mechanism for simultaneous increase in cAMP and cGMP intracellular levels. On the other hand, in cells expressing iNOS, there was a significant decrease in GHRH-induced cAMP production, suggesting that elevated NO and or cGMP could inhibit adenylyl cyclase activity. This hypothesis, however, requires further investigation, because the LPS/IFN treatment could also affect expression of other molecules.

In conclusion, our data show that normal and immortalized pituitary cells express α_1- and β_1-sGC subunits and that basal (in the absence of agonist) NO production is sufficient to activate this enzyme. The results further indicate that G_s-coupled receptors stimulate sGC activity in a dual manner: by stimulating NO production and by phosphorylating the α_1-subunit (Fig. 11). The rise in NO production above basal is not critical for G_s-coupled receptors, but inhibition of basal NOS activity also inhibits agonist-stimulated cGMP production. This observation indicates that the occupancy of NO binding domain is essential for receptor action, *i.e*. receptor-dependent phosphoryla-

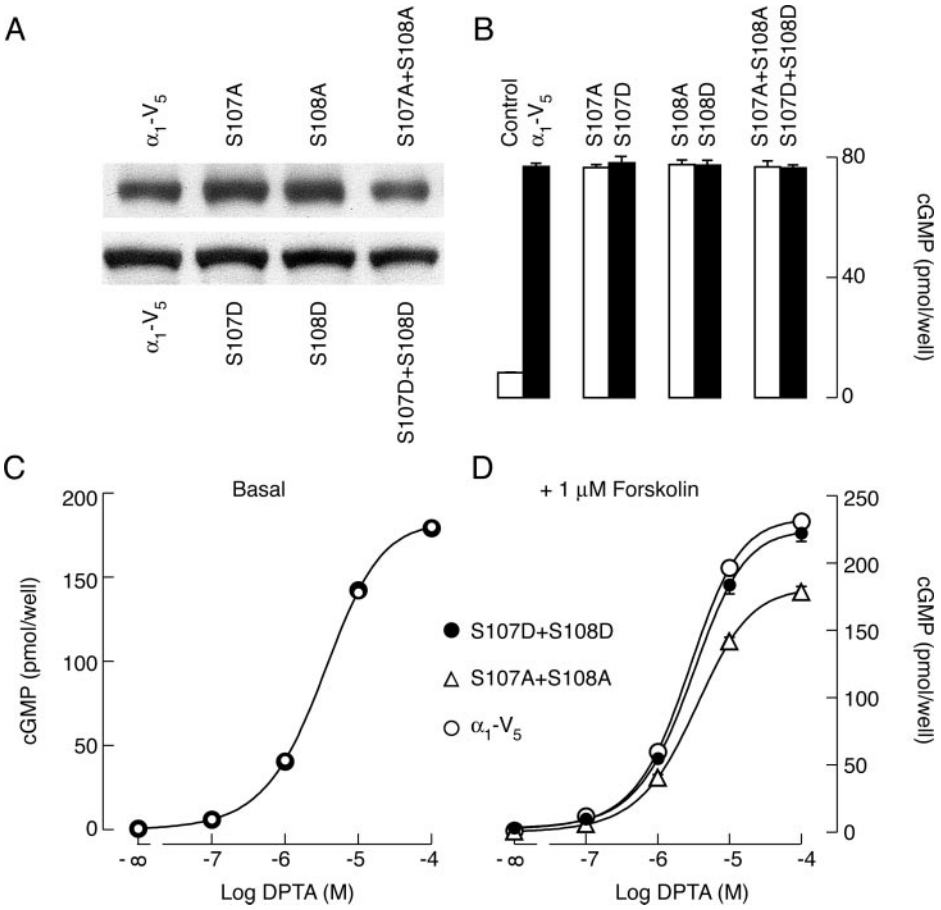

Fig. 10. Comparison between Alanine and Aspartic Acid Mutants of α_1-sGC on the Enzyme Activity

A, The expression levels of α_1-V_5 sGC and its mutants in GH_3 cells, estimated by Western blot analysis, using the specific anti-V_5 epitope antibody as described in *Materials and Methods*. B, The lack of phosphomimic effects of aspartic acid-bearing mutants on basal cGMP production. C, Dose-dependent effects of DPTA on cGMP production in cells expressing α_1-V_5 sGC, S107A+S108A, and S107D+S108D mutants. D, The lack of forskolin (1 μM)-induced augmentation of NO-controlled cGMP production in S107A+S108A mutant (*triangles*) but not in S107A+S108D mutant (*solid circles*). In panels C and D, basal cGMP production was subtracted and basal NO production was inhibited by L-NAME.

tion of α_1-sGC enhances the effectiveness of NO in activating sGC. This mechanism could be important for facilitation of cGMP intracellular signaling functions at steady NO production.

MATERIALS AND METHODS

Cell Cultures and Treatments

Experiments were performed on anterior pituitary cells from adult female Sprague Dawley rats (Taconic Farms, Inc., Germantown, NY) and GH_3 immortalized cells (ATCC, Manassas, VA). Pituitary cells were dispersed as described previously (18). Cells were cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% horse serum, and penicillin (100 U/ml) and streptomycin (100 μg/ml). GH_3 immortalized pituitary cells were cultured in Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 15% heat-inactivated horse serum, 2.5% fetal bovine serum, and gentamicin (100 μg/ml). Adenylyl and guanylyl cyclase was stimulated by GHRH from Bachem California, Inc. (Torrance, CA), forskolin (RBI Signaling Innovation, Natick, MA), and PACAP 27 and VIP (both from Calbiochem, La Jolla, CA). To express iNOS, cells (10^6/well) were treated for 16–18 h with 30 μg/well LPS + 1000 IU/well IFN-γ (LPS+IFN), both from Sigma Chemical Co. (St. Louis, MO). To elevate NO levels, cells were treated with 3,3′-(hydroxynitrosohydrazino]bis-1-propanamine (DPTA) from Alexis Biochemicals (San Diego, CA). Basal NOS activity was inhibited by L-NAME (RBI), whereas 3-isobutyl-1-methylxanthine from Sigma was used to attenuate phosphodiesterases. PKA activity was inhibited by 4-cyano-3-methylisoquinoline (CMI) from Calbiochem.

cGMP, cAMP, and Nitrite Measurements

Cells (1 million per well) were plated in 24-well plates in serum-containing M199 and incubated overnight at 37 C under 5% CO_2-air and saturated humidity. Before experiments, medium was removed and cells were stimulated at 37 C under 5% CO_2-air and saturated humidity for 60 min if not otherwise specified. Cyclic nucleotides were measured in incubation medium (released) and in cell extract using specific antisera provided by Albert Baukal (National Institute of Child Health and Human Development, Bethesda, MD). Re-

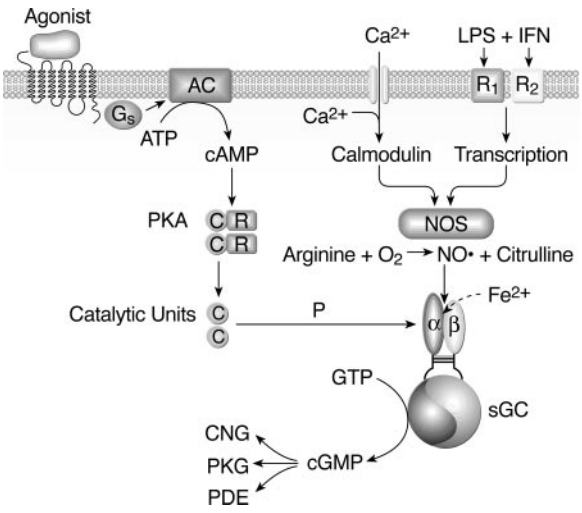

Fig. 11. Schematic Representation of the Relationship between G_s Protein-Coupled Receptors and NO-Controlled sGC Activity

C and R, Catalytic and regulatory units of PKA; P, phosphorylation; R_1&R_2, receptors for LPS and IFNγ; CNG, cyclic nucleotide-gated channels; PKG, protein kinase G; PDE, phosphodiesterase.

sults are shown as combined values of released and cell content cyclic nucleotides. For measurements of NO production, sample aliquots were mixed with equal volumes of Greiss reagent containing 0.5% sulfanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid (all from Sigma), after which the mixture was incubated at room temperature for 10 min and the absorbance measured at 546 nm. Nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite.

RNA Isolation and RT-PCR

Total RNA was extracted from a mixed population of anterior pituitary cells or GH_3 cells using TRIZOL reagent (Life Technologies, Gaithersburg, MD), and its concentration and purity were determined spectrophotometrically. RNA samples were subjected to RT-PCR to determine whether these cells contain transcripts for $\alpha1$, α_2, $\beta1$, and β_2 sGC subunits, as well as for GHRH receptor. To eliminate residual genomic DNA, RNA samples were treated with DNase I. Total RNA (2 μg) from each sample, after DNase I treatment, was reverse transcribed into cDNA in a 20 μl reaction mixture containing oligo (dT)18 primer and Superscript II reverse transcriptase (Life Technologies) according to the supplier's instructions. An aliquot of 5 μl of the reverse transcriptase reaction was amplified with PCR reagent system (Life Technologies) in a final volume of 50 μl containing 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 1.25 U of TaqDNA polymerase. Sequences for sense and antisense primers, respectively, were as follows: α_1sGC, 5′-CGGGGGAGTGTCCTTTCTCC-3′ and 5′-GGTGCTCTTCACGTGGACCG-3′; α_2sGC, 5′-CCAGCCCGGAAGAGGAAGGG-3′ and 5′-CTTTCCTGCAGCCTTGATCATTCCC-3′; β_1sGC, 5′-GATCCGCAATTACGGTCCCG-3′ and 5′-TGGAGAGGGATGTCACTCAG-3′; β_2sGC, 5′-GACAGGATGCTGCGGACACTT-3′ and 5′-TCGACCCATAGTCTCTCAGGA-3′; and GAPDH, 5′-GGCATCCTGGGCTACACTG3-′ and 5′-TGAGGTCCACCACCCTGTT-3′. Sequences for sense and antisense primers for GHRH receptors were: no. 2, 5′-TTGCTGAACCTGTGGGGAGTTG-3′ and 5′-GGGTCTGAGCCAAAATGAGAGAAG-3′; no. 3, 5′-TTGCTGAACCTGTGGGGAGTTG-3′ and 5′-TTGATGATCCACCAGTAGGGGG-3′; no. 4, 5′-ATCAAGAGGTGAGGACGGAGATT-3′ and 5′-AAGTCG GAG GTTGGTAT-3′; no. 5, 5′-CATCTCCTAGGTCCAAACCAGC-3′ and 5′-GAAGTTCAGGGTCATGGCCATA-3′ (see Fig. 1A). The PCR products were analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide. Reactions without reverse transcriptase served as negative controls.

Plasmid Preparation and Transfection

The coding sequence for the α1-sGC (46) was amplified by PCR. The PCR product was directly cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, San Diego, CA) in the reading frame of V_5 epitope, to add it to the C terminus of α1-sGC. The size and orientation of insert were verified using the restriction enzyme digestion method. The sequence was confirmed by automated sequencing (Veritas, Inc., Rockville, MD). For point mutations, six serine residues localized on α_1-sGC (107, 108, 241, 359, 360, 541) were selected following the most common phosphorylation RXS* motif for PKA. The residues were mutated to alanine and aspartic acid, producing single- or double-mutant constructs. This was done using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and the mutations were confirmed by automated sequencing. Expression vectors for constitutive active PKA (C_{QR}) and negative-dominant PKA mutant (REV_{AB}) were kindly provided by Dr. G. S. McKnight (University of Washington, Seattle, WA). Large-scale plasmid DNA for transfection was prepared using the Plasmid Maxi Kit (QIAGEN, Valenca, CA). GH_3 cells were transfected by Lipofectamine 2000 reagent (Invitrogen) following the manufacture's instructions and using 2 μg DNA per well in 2 ml serum-free OptiMEM (Invitrogen). After 6 h of incubation, transfection mixture was replaced with normal culture medium. Cells were subjected to experiments 36–40 h after transfection.

Immunoprecipitation

Pituitary and GH_3 cells were preincubated with CMI for 15 min and stimulated with 1 μM forskolin or 100 nM GHRH for 60 min. Cells were washed twice with ice-cold PBS and lysed in 1-ml buffer containing 20 mM HEPES, 10 mM EDTA, 40 mM β-glycerophosphate, 1% tergitol, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and a cocktail of phosphatase inhibitors (0.05 mM (−)-P-bromotetramisol oxalate, 10 μM cantharidin, and 10 nM microcystin LR, pH 7.5; Calbiochem). Cell lysates were centrifuged at 12,000 $\times$ *g* for 20 min at 4 C, and protein concentration in supernatant was estimated by the Bradford method using BSA as a standard. An equal amount of protein in 1 ml of supernatant was mixed with anti-sGC polyclonal antibody (Calbiochem) or anti-V5 epitope monoclonal antibody (Invitrogen), or polyclonal anti-nNOS (Cayman Chemical, Ann Arbor, MI). Overnight incubations were carried out at 4 C with constant rotation. Immunoprecipitated complexes were recovered using 30 μl protein G or protein A agarose resin slurry (1:1) (Oncogene Research Products, San Diego, CA) for an additional incubation at 4 C overnight. Precipitated proteins were washed three times with 1 ml lysis buffer, denatured for 5 min at 95 C, and loaded on 4–12% SDS-PAGE gradient gel (Novex, San Diego, CA).

Western Blot Analysis

Protein concentration was estimated by the Bradford method using BSA as a standard. Equal amounts of lysates were run on one-dimensional SDS-PAGE, using a discontinuous buffer system (Novex), and proteins were transferred to a polyviny-

lidine difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA), using a wet transfer, following the manufacturer's recommendation. The immunodetection of sGC was done with an antibody that recognizes both α_1- and β_1-subunits (Calbiochem) or, in the case of cells transfected with V_5-tagged α_1-sGC, with anti-V_5 epitope antibody (Invitrogen). The phosphorylated sGC was detected using a monoclonal antiphosphoserine antibody clone 16B4 (Calbiochem), and, for detection of phosphorylated nNOS, monoclonal antiphosphothreonine antibody clone 4D11 (Calbiochem) was used. Anti-PKA_C (Transduction Laboratories, Inc., Lexington, KY) was used to detect expression of PKA_C and its C_{QR} mutant. The secondary antibodies were a goat antirabbit IgG or antimouse IgG-IgM (Kirkegaard and Perry Laboratories; Gaithersburg, MD). Both antibodies were linked to the horseradish peroxidase. The reactive bands were always determined with a luminol-based kit (Pierce Chemical Co., Rockford, IL), and the reaction was detected by an enhanced chemiluminescence system, using x-ray film. The immunoreactive bends were analyzed as three-dimensional images using a GS-700 Imaging Densitometer (Bio-Rad Laboratories Inc., Hercules, CA). The OD of images is expressed as volume (OD $\times$ area) adjusted for the background, which gives arbitrary units of adjusted volume.

Calculations

cAMP and cGMP data are shown as total (cell content + medium) nucleotide levels. The results shown are means $\pm$ SEM from sextuplicate determination in one of at least three similar experiments. *Asterisks* indicate a significant difference among means, estimated by Student's *t* test.

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